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General

The promise of cytokine antibody arrays in the drug discovery process

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The introduction of cytokine antibody arrays has added a new approach for investigators to simultaneously measure multiple cytokine levels in biological samples. Several different platforms have been developed. The ability to measure hundreds of cytokine levels with high specificity and sensitivity within a very limited amount of samples is a powerful tool. Many investigators worldwide have applied this novel technology in their biomedical research, particularly in drug discovery. Undoubtedly, the technology will continue to be improved and the application increased in the next several years.

Keywords: antibody array, cytokine, drug discovery, protein array

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1. Introduction

Cytokines, including chemokines, growth factors, angiogenic factors and proteases play critical roles in many cellular events. Data from clinical and animal studies strongly suggest the involvement of cytokines in the development of many common diseases, particularly atherosclerosis, cancer, AIDS and allergy. Therefore, cytokines are important targets in drug discovery.

Identification of cytokine expression levels is a common experimental procedure in drug discovery. Enzyme-linked immunosorbent assay (ELISA) is the most common method used to measure cytokine expression levels due to its high specificity and sensitivity [1,2]. Although this methodology is well-suited for single-cytokine analysis, it is much more desirable to simultaneously measure multiple cytokines from a relatively small sample size in a rapid fashion. This is particularly important in drug discovery.

An emerging technology that meets this demand is cytokine antibody arrays. In this approach, antibodies against cytokines are immobilised onto the surface of solid supports. The cytokines in biological samples are then captured on the surface of arrays after incubation. The captured cytokines are detected using a variety of methods. By using this approach, hundreds of cytokines can be detected. Many different platforms of cytokine antibody arrays have been developed and are commercially available. Hundreds, if not thousands, of investigators around the world have started to apply these novel technologies in drug discovery. The promise of cytokine antibody arrays in drug discovery has been gradually realised. This review focuses on the discussion of cytokine antibody array technologies and their applications in drug discovery.

2. Implication of cytokines in drug discovery

Cytokines play a central role in a wide range of physiological processes, such as immune response, inflammation, cell growth, differentiation, apoptosis, wound

healing, signal transduction and homeostasis [3]. Most cytokines exist in Drosophila and primitive vertebrates from comparative phylogenetic analysis [4], suggesting the importance of cytokines in normal physiology. Compelling data either from clinical observations or laboratory animal models indicate the involvement of cytokines in the development of many common and life-threatening diseases, including cardiovascular disease, allergic inflammatory disease, transplantation, neuroinflammtion, cancer and HIV-associated disease [4]. The alteration of cytokines in many diseases makes this an important target for drug development and a biomarker for personalised medicine, particularly in oncology, autoimmune disease and arthritis (**Table 1**) [5]. A number of cytokines have been used as targets for drug development, such as TNF-α and IFN-γ [5-7]. Several strategies have been used for development of cytokine-based therapy, among them, antibody therapy, small molecules and recombinant protein are the most common approaches for targeting cytokines [8-10]. This is a very significant and rapidly growing market.

Conventionally, cytokines are primarily measured by biological activity assays and immunological assays, such as ELISA and radioimmunoassay. Although ELISA is popular and has been widely used, it only measures one cytokine during one experiment. When large numbers of cytokines need to be analysed, it becomes expensive, time-consuming and even impossible to accomplish due to the limited amount of biological samples.

Many cytokines are pleiotropic, possessing overlapping functions and regulating the production of other cytokines. To a certain degree, almost all cytokines have effects that are synergistic with or antagonistic toward other cytokines. Consequently, the make-up of the cytokine milieu is often of greater importance than the actions of a single cytokine. In addition, accumulating evidence suggests that a complex cytokine network is involved in many diseases such as cancers [11,12]. Autocrine and paracrine cytokine loops influence the biology of cancer [13,14]. Cytokines not only provide defense against cancer cells, but also promote cancer cell growth at every stage of cancer development [15]. Accumulating data suggest that abnormal cytokine expression is linked to cancer progression [16,17], response to chemotherapy [18,19] and metastatic status [20,21]. Obviously, measurement of one cytokine level in a biological system or disease status provides only a fraction of the information related to the existing physiological and pathological state. Therefore, highly paralleled assays to simultaneously detect the expression of multiple cytokines at any given time point will provide a more realistic indication of the complexity of the cytokine network.

3. Approaches to detect expression levels of multiple cytokines

The limitation of conventional approaches such as ELISA to detect only one cytokine has prompted the research community to develop more highly parallel approaches to measure multiple cytokine levels. Among the most promising technologies developed are antibody arrays, fluorescent beads, aptamer arrays and eTag (**Table 2**).

The fluorescent bead-based approach has been available for a number of years [22-24]. The core technology is based on microspheres (beads) coded with different ratios of two fluorescent dyes. The specific ratio of each microsphere creates a unique spectral signature, which can be determined by its fluorescent ratio. In addition, each set of beads is coated with a specific capture antibody. In an immunoassay, different sets of beads are incubated with biological samples in a single reaction. The cytokines, which are bound to the antibody-coated-beads, are detected with fluorescently labelled detection antibodies. The signal intensities are measured in a flow cytometer, which is able to quantitatively measure the amount of captured targets on each individual bead. Therefore, multiple cytokines can be determined from a single experiment. The bead-based approaches have several advantages. Because the reaction is carried out in a solution, to detect a particular group of cytokine expression levels, one can simply add the corresponding quality-controlled beads in the system. Furthermore, no washing steps are required when performing the assay. The sample is incubated with the particular group of beads together with fluorescently-labelled detection antibodies. The formation of an immuno-complex is measured by the flow cytometer. The major disadvantage of this approach is the difficulty in achieving high-density multiplexed detection and the high cost of producing coded beads with specific antibodies. The current technology allows only up to 100 cytokines to be detected.

Another approach to detect multiple cytokines is eTag, developed at Aclara [25,26]. The eTag assay system combines proprietay eTag reporters and capillary array electrophoresis (CE). The eTag reporters are distinct fluorescent small-molecules, each of which has unique migration properties in CE assay. Each antibody is attached with an unique eTag. A set of eTag-antibodies, therefore, can be used to monitor multiple cytokines. Following binding of eTag-antibodies to cytokines, eTags are released and quantitated by CE assays. The ability to detect both protein and mRNA side-by-side on the same sample is the major advantage of the eTag approach. Shortcomings of this approach include low density, difficulty in generating specific eTag antibodies and complicated experimental procedures, which may not be familiar to many researchers.

The expression levels of cytokines can also be measured by Aptamer arrays [27,28]. Aptamers are oligonucleotides that bind to proteins with high specificity, affinity and diversity. Aptamer arrays are generated by spotting different aptamers onto surface of solid support. The aptamer chips are incubated with biological samples. The cytokines in samples are captured by their corresponding aptamers. After UV cross-linking aptamer to protein, cytokine expression is detected by protein staining. At this moment, only a limited number of aptamers are available for binding to specific

Table 1. Examples of cytokines in diseases.

Disease associated with cytokine alterations	Allergy, psoriasis, mastocytosis, cancer, colitis, AIDS, autoimmune diabetes, multiple sclerosis, glaucome, asthma, obstructive pulmonary disease, rheumatoid arthritis, systemic lupus erythematosis, graft rejection, infection, sepsis, atherosclerosis, hypertension, ischaemia–reperfusion, Crohn's disease, Grave's and Hashimoto's thyroiditis
Cytokines implicated in diseases	IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-13, IL-18, IL-23, IL-25, IFN-α, IFN-γ, FasL, TGF-β, VEGF, NGF, EGF, TNF-α, MCP-1, MIP-1α, RANTES
Strategies for targeting cytokine therapy	Active antibody therapy, passive antibody therapy, chemical inhibitors, receptor antagonists, fusion protein, recombinant protein
Drugs targeting cytokines	Infliximab, Adalimumab, CDP571, PEG Fab, Etanercept, Lenercept, Antiferon, Lerdelimumab, B-N10

AIDS: Acquired immune-deficiency syndrome; EGF: Epidermal growth factor; FasL: Fas ligand; IFN-: Interferon; IL: Interleukin; MCP: Macrophage chemotactic protein; MIP: Macrophage inflammatory protein; NGF: Neuronal growth factor; RANTES: Regulated upon activation, normal T cell expressed and secreted; TNF: Tumour necrosis factor.

Table 2. Comparison of several major methods of multiplexed cytokine profiling.

	Antibody arrays	**Fluorescent beads**	**eTag**	**Aptamer arrays**
Common feature	Simultaneous detection of multiple cytokine levels in a single experiment with a minute amount of sample			
Principle	Antibodies are immobilised in array format onto a solid support. Cytokines binding to corresponding antibodies are detected and the positions in the arrays indicate the specific cytokines.	Beads are coded with different ratios of two fluorescent dyes and specific antibody. The cytokines bound to the beads can be measured by flow cytometry.	Antibodies are labelled with different eTag. Upon binding, eTGA will be released and can be detected using capillary electrophoresis.	Aptamers are immobilised in predetermined positions onto a solid support. Cytokines captured by aptamers can be detected by protein staining.
Major advantage	Flexible, high-density, low cost	Fast, no wash-required	Different sample source, both protein and RNA can be run side by side	Simple protein staining, high specificity
Major player	RayBiotech, Pierce, S&S	Luminex, BD Biosciences	Aclara	SomaLogic
Major disadvantage	Difficult to set up automation	Relative low density	Complicated experimental procedures Difficult to generate eTag protein Relative low density	Difficult to generate cytokine-bound aptamer
Major equipment	Film processor on laser scanner	Flow cytometer	Capillary electrophoresis	Film processor on laser scanner
Skill required	Some	Some	High	Some
Volume required	50 – 500 μl	50 – 500 μl	50 – 500 μl	50 – 500 μl
Quantification	Semi-quantitative to quantitative	Quantitative	Semi-quantitative	Semi-quantitative

cytokines. However, aptamers, which are stable and easy to handle, can be synthesised, thereby providing an unlimited source and making standardisation procedures easy. The use of photo-crosslinkable aptamers allows the covalent attachment of aptamers to their cognate proteins with very low backgrounds from other proteins in body fluids. Furthermore, proteins can be directly detected using any reagent or procedure, which distinguishes functional groups of amino acids from those of nucleic acids (and the solid supports). Therefore, aptamers are very attractive capture reagents for protein array applications [29]. SomaLogic is developing aptamer arrays for protein detection.

Two-dimensional (2-D) electrophoresis coupled with mass spectrophotometer can also be used to detect multiple cytokine expression levels in an unbiased base. However, due to the low-detection sensitivity and low abundance of cytokines, the 2-D approach is barely used in the detection of cytokine expression.

4. Antibody arrays for detection of multiple cytokine expression levels

An emerging new technology for the detection of multiple cytokine expression levels is cytokine antibody arrays. The technology has been developed in many academic research labs and commercial biotech companies. Indeed, cytokine antibody arrays represent the most advanced and diversified antibody array technology. **Table 3** lists available cytokine antibody array commercial vendors.

Ekins has developed a theory that immunoassays in microarray format, such as cytokine antibody arrays in general, should be considered ambient analyte assays and the microarray nature will allow to achieve high sensitivity [30-32]. Such theoretical analysis has been demonstrated experimentally that sandwich-based cytokine antibody arrays fulfill the criteria of ambient analyte regimen [33].

Several different platforms of cytokine antibody arrays have been developed. Membrane-based cytokine antibody arrays [34-37] have the advantages of being flexible, easy to perform and requiring no sophisticated equipment. This approach is gaining popularity among investigators [38,39]. In this platform, capture antibodies are spotted onto membranes, the array membranes are incubated with biological samples, and the bound cytokines are then detected with detection antibodies. The signals can be measured by chemiluminescence, fluorescence or colour detection. Anyone who is familiar with the western blot procedure can easily perform the array experiment without additional training. The major limitation for membrane-based approach is the difficulty associated with adapting to automation.

In the tissue culture plate format of cytokine antibody arrays [40,41], capture antibodies are arrayed onto the bottom of a 96-well plate and subsequently the experiment can be carried out in the plate. Therefore, the major advantage of this format is that the experiment can be adapted to a liquid handling system. However, the limited surface of 96-well plates greatly reduces the production of higher density arrays.

Because most DNA arrays are constructed on glass slides, cytokine antibody arrays also have been developed using a glass slide format by many groups [42-46]. Like DNA arrays, capture reagents, in this case antibodies, are spotted onto the surface of a glass slide in array format. The experiment can then be performed using existing DNA array instruments such as laser scanner.

The expression levels of cytokines can be determined and compared among samples like DNA arrays. In addition, signal intensities can be compared with standard proteins; therefore, the exact expression levels of cytokines can be measured quantitatively.

Almost all cytokine antibody arrays have been developed based on the sandwich ELISA principle, where two antibodies are required. One antibody serves as capture and another antibody functions as detection. Because this approach requires a detection antibody, it significantly increases the complexity in the development of cytokine antibody arrays and greatly restrains the likelihood of development of high-density cytokine antibody arrays. Recently, Dr Lin has developed a biotin-labelled-based approach in which biological samples are labelled with biotin. The biotin-labelled samples are then incubated with an antibody array chip and the captured proteins are detected using fluorescence-labelled streptavidin. Furthermore, the two-color approach can be adapted to biotin-label-based cytokine antibody arrays. Obviously, the label-based approach can be used to develop very high-density arrays cost-effectively. The label-based antibody arrays using cy3 and cy5 were developed in Dr Haab's group [47]. However, there is a potential problem inherent in the labelling process. Not only can the labelled protein's behaviour change, but it is also difficult to attain uniform and consistent labelling for every protein. Furthermore, the approach only allows for semiquantitative measurement of cytokine levels. Li *et al.* [48] have carefully compared these two approaches and concluded that the ELISA-based approach generally has high sensitivity compared with the labelled-based approach.

To enhance the detection sensitivity of cytokine antibody arrays, two approaches can be applied. The rolling circle amplification (RCA) system [45] utilises DNA amplification for the detection of antibodies bound to antigens [49-52]. An oligonucleotide primer is covalently attached to an antibody. The antibody-DNA conjugate then binds to the antigen that is captured on the surface of cytokine antibody chips. A DNA circle then hybridises to its complementary primer on the conjugate. The resulting complex is washed to remove excess reagents, and the DNA tag is amplified by RCA in the presence of DNA polymerase and nucleotides. The RCA results in a DNA molecule consisting of multiple copies of the circle DNA sequence that remains attached to the antibody. The amplified DNA can be detected by hybridisation with fluorescence-labelled complementary oligonucleotide

Table 3. Commercial vendors for cytokine antibody array kits.

	RayBiotech	Pierce	S&S	EMD	Beckman
Platform	Sandwich Label	Sandwich	Sandwich	Sandwich	Oligonucleotide Conjugated antibody Sandwich
Solid support	Membrane	96-well plate	Glass slide	Glass slide	96-well plate
Detection	Chemiluminescence Fluorescence colorimetry	Chemiluminescence colorimetry	Fluorescence colorimetry	Fluorescence colorimetry	Fluorescence colorimetry
Density (multiplex)	≤ 120	≤ 16	≤ 18	≤ 10	≤ 12
Species	Human, mouse, rat	Human, mouse, rat, primate, porcine	Human, mouse	Mouse	Human
Quantification	Semi-quantitative	Quantitative	Quantitative	Quantitative	Quantitative
Sensitivity	pg/ml to ng/ml	pg/ml to ng/ml	pg/ml to ng/ml	pg/ml to ng/ml	pg/ml to ng/ml
CV (intra)	10%	10%	10%	10%	10%
Major equipment	None or film process or imaging system	Imaging system attached to microscope	Laser scanner	Laser scanner	CCD-based A^2 microarray reader
Major advantage	Highest density, flexible, easy to perform, no special equipment required	The experiments can be adapted to a liquid handling system	Easy for DNA microarray facility	Easy for DNA microarray facility	Flexible, automation

CCD: Charge-coupled device; CV: Coefficient of variation.

probes. Although this RCA step greatly increases the detection sensitivity, its widespread application is still very limited due to the complicated procedures, high cost and lack of uniform amplification.

Alternatively, the signals can be amplified using a tyramide-based avidin/biotin signal amplification system. This approach has been demonstrated to be able to detect multiple cytokines at sub picogram levels [44]. However, the amplification process often increases the background. Furthermore, not all antibody signals can be amplified evenly.

5. Applications of cytokine antibody arrays in the drug discovery process

Cytokine antibody arrays have gained popularity over the last several years, with hundreds of reports in the literature of their applications. Here, the application of cytokine antibody arrays in terms of drug discovery will be discussed, but these methods also have many important applications in basic research.

Drug discovery is a time-consuming process and includes target discovery, target validation, lead identification, lead validation, lead optimisation, preclinical study, and finally clinical studies. As the importance of cytokines in normal physiology and disease development is well known, cytokines are important drug targets. The value of the application of antibody arrays to the drug discovery process is now well-recognised because proteins are the sites of drug action. Because novel high-throughput biological applications in the drug discovery process and disease diagnosis require a highly parallel, miniaturised device technology applied to proteins and their biochemical pathways, cytokine antibody arrays meet this increasing demand. Substantial savings could be made both in terms of time and precious reagents by multiplexing and miniaturising these assays. Cytokine antibody arrays can also be used for the determination of off-target effect of cytokine-based therapy because its ability to screen a large number of cytokines including targeted cytokines and off-targeted cytokines simultaneously.

5.1 Identification of potential target

Identification of key factors associated with disease development provides a bona fide target for drug discovery. Inhibition of particular factors associated with certain disease provides rationale to treat the disease.

Because alterations in cytokine levels have been implicated in many life-threatening diseases, including HIV, cancer, asthma and arthritis, the identification of cytokines associated with certain diseases may provide viable targets for treatment of these diseases. One of the most exciting applications of cytokine antibody array technology is to identify key cytokines associated with certain cancer development processes.

Recently, Dr Vargas [53] successfully applied cytokine antibody arrays with the goal to identify potential therapeutic targets of autism, which is a neurodevelopmental disorder with unknown cause. They analysed cytokine expression levels in fresh-frozen tissues from seven patients and cerebrospinal fluid (CSF) from six living autistic patients. The cytokine

profiling clearly indicated that macrophage chemoattractant protein (MCP-1) and tumour growth factor-beta 1 (TGF β1) were the most prevalent cytokines in the brain tissue samples, and MCP-1 was markedly increased in the CSF samples. Their findings suggest that innate neuroimmune reactions play a pathogenic role in an undefined proportion of autistic patients and provide a potential target for further therapies.

With the goal to identify key factors for human breast cancer progression, Lin *et al.* [54] screened a panel of cytokine expression levels in different human breast cancer cell lines with distinct phenotypes using human cytokine antibody arrays. A key factor, IL-8, associated with breast cancer invasion and angiogenesis was identified. A series of follow-up experiments validated the importance of IL-8 in the development of breast cancer.

Autosomal dominant polycystic kidney disease (ADPKD) is a prevalent human genetic disease. Of ADPKD patients, > 50% developed hepatic cysts, which are the most common extrarenal manifestation and are a significant source of morbidity in ADPKD. To identify the autocrine and paracrine factors secreted into the cyst and potential therapeutic target for ADPKD, Nichols *et al.* [55] applied cytokine antibody arrays to analyse human liver cyst fluid and found that epithelial neutrophil-activating protein 78 (ENA 78), IL-6, IL-8 and vascular endothelial growth factor (VEGF) were significantly increased in ADPKD patients' liver cyst fluid compared with non-ADPKD bile. The results were further confirmed and quantitated by ELISA. Furthermore, ADPKD liver cyst cell cultures also released IL-8 and VEGF, suggesting that these factors may serve as autocrine and paracrine factors to direct errant growth of ADPKD liver cyst epithelia, and interruption of these signalling pathways may provide therapeutic targets for inhibiting liver cyst growth.

5.2 Study of drug actions

Another distinct use of cytokine antibody arrays is to identify the molecular mechanisms of poorly understood drugs or other bioactive compounds. The cytokine expression patterns in response to drugs or bioactive compounds can be determined using cytokine antibody arrays. These unique expression patterns may tell us the possible mechanism of certain drug action.

Turtinen *et al.* [56] used cytokine antibody arrays to analyse the profiles of cytokine release from THP-1 monocytes exposed to different amphotericin B (AMB) drug delivery systems. AMB, a polyene antifungal antibiotic has been the 'gold standard' for the treatment of systemic fungal infections. The data showed that Fungizone (FZ) and Amphotec (ABCD) specifically induce significant release of more inflammatory cytokines such as TNF-α, IL-8, growth related oncogene, MCP-1, regulated upon activation, normal T cell expressed, and presumably secreted (RANTES), IL-10 and IL-6 than either AmBisome (L-AMB) or Alelcet (ABLC). Interestingly enough, the expression patterns are well-correlated with clinical side effects of AMB. According to cytokine expression profiles, AMB delivery systems fall into two groups, with FZ and ABCD in one group and L-AMB and ABLC in the other group. These results may help to explain the different side effects observed with FZ and ABCD and with L-AMB and ABLC. Furthermore, the data may also provide rationales for the screening of antifungal drugs with fewer clinical side effects.

In another interesting study, cytokine antibody arrays have been used to determine potential physiological differences between free- and triglyceride-type conjugated linoleic acid [57]. These studies show that there is a slight but significant difference between the functionalities of free- and triglyceride-type conjugated linoleic acid as determined by the different cytokine expression pattern using cytokine antibody arrays.

Vitamin E is an antioxidant with diverse biological function. To study the potential vitamin E action, Lin *et al.* [58] applied cytokine antibody arrays to screen a panel of cytokine profiles from patient plasma before and after vitamin E supplementation. The array data showed that supplementation of vitamin E decreased the expression levels of a number of chemokines, including MCP-1, ENA-78, IL-1α, monokine induced by gamma interferon, RANTES and TNF-β. Interestingly enough, these chemokines have been linked to cancer and cardiac diseases. This finding was further confirmed by *in vitro* studies showing that indeed, vitamin E treatment significantly downregulates MCP-1 expression. Because the preventive effects of vitamin E on certain types of cancers and cardiac diseases has been illustrated, this study may provide valuable information about molecular mechanisms of vitamin E supplementation in the chemoprevention and identification of novel targets.

5.3 Personalised medicine

Another attractive application of cytokine antibody arrays is in clinical proteomics studies. With the ability to simultaneously measure larger numbers of cytokine levels in single patient samples, cytokine antibody arrays have been used to determine cytokine profiles for diagnosis, subclassification, prognosis, disease prediction and an understanding of intermediate steps leading to the disease. For instance, the expression profiling of cytokines may enable separation of disease into several categories, which correlates with survival. Molecular classification of tumours according to gene expression patterns [59-62] using cDNA microarrays are a hot research area. The approach using cytokine antibody arrays, if successful, will have an even more profound effect and practical significance than the cDNA microarray approach because analyses using cytokine antibody arrays can be easily performed using serum, plasma, amniotic fluid, sputum, CSF, synovial fluid and urine.

Cerebral palsy (CP) is a major neurodevelopmental disability in childhood. The cause of this disease is poorly understood. To identify a potential biomarker for CP, Dr Kaukola *et al.* [63] profiled 78 cytokines on 19 CP children and 19 gestation-matched paired controls using cytokine antibody arrays coupled with RCA. Their studies found that 11 biomarkers

correlated with the length of gestation, both in cases and controls. Expression levels of IL-6, IL-8 and plasminogen activator receptor declined with increasing gestational age. The cytokine patterns at birth differ between premature and term infants who develop CP. This is the first article that reports on biomarker differences between CP cases and controls in both preterm and term children.

Identification of markers for early detection and predictive purposes will have a significant impact on drug discovery. Celis *et al.* [64] attempted to identify a marker for breast cancer patients. To reach this goal, they screened cytokine expression in the nipple aspirate fluid, which contains proteins directly secreted by the ductal and lobular epithelium, in patients with breast cancer using cytokine antibody arrays and other proteomic technologies. Their results clearly showed a change in the expression profiles of a group of cytokines. Because such tumour interstitial fluid perfuses the breast tumour microenvironment and may provide a novel and potentially highly promising source of biomarkers, further analysis of additional samples from patients may identify clinically useful biomarkers.

In another study, Pockaj *et al.* [65] analysed cytokine profiles using cytokine antibody array technology from breast cancer patients' sera at different stages of cancer development. Their data demonstrated a distinctive cytokine expression profile in breast cancer patients at different stages, suggesting a potential application of cytokine antibody arrays in diagnosis of different stages of breast cancer.

Cytokine antibody arrays have also been used to identify the potential expression pattern for ovarian cancer [66]. The expression levels of 43 cytokines from 13 ovarian cancer patients and 12 normal female controls were determined using cytokine antibody arrays. The data were analysed using t-test and two-way hierarchical cluster analysis approaches. The studies identified 22 cytokines with significantly increased expression levels in ovarian cancer patients compared with controls. Cluster analysis suggested an interesting link between cytokine profile and ovarian cancer.

Tadros *et al.* [67] extended the analysis of cytokine profiles by combination of laser capture-microdissection and cytokine antibody arrays. They successfully determined the cytokine expression using laser capture-microdissected urothelial cells from ureteropelvic junction obstruction patients. Several cytokines, including GROα, IL-1α, INF-γ, IL-8, TNF-α, RANTES, and macrophage inflammatory protein-1β were significantly increased in hyperplastic urothelial cells compared with paired control cells, whereas TGF-β and IL-10 were markedly reduced. The expression pattern of cytokines identified in cytokine antibody arrays provides potential biomarkers and a starting point to investigate the molecular mechanism responsible for the development of hyperplastic urothelial cells. The study clearly demonstrated that cytokine antibody arrays are robust enough for analysis of laser capture-microdissected clinical samples.

Other studies [68] have also attempted to discover biomarker classification for immunodeficiency, ulcerative colitis, Crohn's disease and autism. The application of cytokine antibody arrays in molecular classification is a logical extension from DNA chip experience and appears attractive; however, more data are needed in order to adjust the practical application of this approach.

5. 4 Investigation of molecular mechanism of cellular events

The development of disease is a complex process and involves in many changes in molecules and signal pathways. It is critical to understand the molecular mechanisms of disease development because elucidation of molecular mechanisms will provide new targets for drug discovery, help us in better drug design and enhance our understanding of drug action. Cytokine antibody arrays have been widely used in the investigation of molecular mechanisms of disease development.

A Merck research team presented a classic example of how cytokine antibody arrays can be used to pinpoint unknown disease development mechanisms [69]. It is well-known that leukotriene B4 (LTB4), a product of the 5-lipoxygenase pathway of arachidonic metabolism, is involved in atherosclerosis. However, the molecular mechanisms for the atherogenic effect of LTB4 are not well understood. Using cytokine antibody arrays, the group found that MCP-1 was strongly induced by LTB4 in primary human monocytes. The induction of MCP-1 by LTB4 identified by antibody arrays was confirmed by ELISA and real-time PCR. They further exploited the signal pathway leading to the induction of MCP-1 by LTB4. Their studies provide evidence demonstrating for the first time that LTB4 strongly induces MCP-1 production in primary human monocytes and linking the pathway leading to the development of atherosclerosis.

HIV Type 1 (HIV-1) infection can lead to HIV-1-induced encephalopathy. It has been proposed that host products from HIV-1-infected macrophages, which become activated as a result of infection, may contribute to the development of encephalopathy. To define the potential mechanism of this process, Xu *et al.* [70] determined the expression of cytokines from macrophages in response to pg120. They identified host factors that were upregulated from infected macrophages that may contribute to the encephalopathy. They demonstrated that the human CC chemokine I-309, monocyte chemoattractant proteins (MCP-2, MCP-3), ILs (IL-3, -5, -6, -7, -8), RANTES, granulocyte/macrophage colony-stimulating factor and growth-related oncogene (GRO) were elevated in infected compared with uninfected macrophages, with IL-5 and IL-6 being increased the most robustly. It is likely that I-309 and MCP regulate migration of peripheral blood mononuclear cells through the blood–brain barrier, whereas RANTES and other cytokines may be involved in cellular communication, survival and differentiation.

Schweitzer *et al.* [45] applied cytokine antibody arrays coupled with immuno-RCA detection methods to identify the key factors during maturation of Langerhans cells (LCs). Upon stimulation with lipopolysarcharide or TNF, 51 cytokines in dendritic cells were measured. In agreement with early works, several new cytokines were also found to be regulated upon stimulation. For example, the chemokine I-309 was shown for the first time be secreted by dendritic cells. This analysis was useful for characterising LC activity during differentiation.

In addition, human cytokine antibody arrays have been used to study physiological processes. Giroux and Denis [71] have identified a novel T cell subset that has the ability to recruit different leukocytes of the innate immune system upon Fas engagement. To define the potential role of this novel T cell subset, they screened 120 cytokine expression and found that this type of cell secreted CCL1, 5, 7, 8, 17 and 27, CXCL1, 2, 3, 8, 11 and 12 following Fas engagement. Further characterisation of signalling mechanisms involved in chemokine secretion might allow researchers to identify novel therapeutic targets for controlling inflammatory diseases.

Transdifferentiation of stem cells shows promise for use in tissue repair medicine. Mesenchymal stem cells (MSC) appear useful for applications in repair medicine. To determine whether or not cytokines produced by MSC were involved in the allogeneic response of MSC, Potain *et al.* [72] applied human cytokine antibody arrays to examine the production of cytokines from MSC. They were able to identify a group of cytokines that were expressed in MSC. The cytokine antibody array data help to explain that, in the absence of an immune challenge, MSC might produce a balanced group of soluble factors to maintain homeostasis.

6. Expert opinion and conclusion

6.1 Detection of cytokines by protein array technology

Because the importance of cytokines in cell events and disease development is well-known and cytokine antibodies and recombinant proteins are widely available, detection of multiple cytokine expression levels by cytokine antibody array technology has emerged as a novel approach. The most common way to detect cytokines by protein array technology is sandwich-based cytokine antibody arrays. This approach is based on the conventional ELISA principle. It has been demonstrated repeatedly that cytokine expression can be measured by this approach with high specificity, sensitivity and reproducibility. The major limitation for this approach is the difficulty in expanding the density of arrays (increasing the number of cytokines to be measured in the same arrays).

To overcome this limitation, cytokine expression can also be determined by label-based antibody arrays. In this approach, samples are first labelled with a detection molecule such as biotin and fluorophore. The labelled cytokines are then exposed to cytokine antibody chips. Finally, cytokines bound to the arrays can be detected based on the labelled molecule. Obviously, this approach allows investigators to develop high-density cytokine antibody arrays. The major drawbacks are that the detection sensitivity and specificity is usually lower than the sandwich-based approach. Furthermore, different cytokines or the same cytokines, but in different environments may be labelled with different efficiency, and the labelled cytokines may behave differently with unlabelled counterparts.

This limitation may be resolved by unlabelled approaches, such as surface plasmon resonance [73], planar waveguide technology [74], electrochemical detection [75] and microcantilevers [76]. If those approaches turn out to be sensitive enough to detect cytokine expression and the cost is low enough for use in general laboratories, it will represent a major advance in detection methods.

On the other hand, cytokine expression can also be detected by reversed phase protein array technology [77]. In this approach, samples containing different cytokines are spotted onto a solid support. The arrays are exposed to a mixture of antibodies, each of which is labelled with a distinct fluorophore or quantum dot. Multiple cytokines can be detected. In summary, at least four different approaches can be applied to profile cytokine expression using protein array technology as shown in **Figure 1**. In the short term, the sandwich-based approach will be a predominant method for cytokine profiling. As the technology advances, unlabelled approaches will become welcome.

6.2 Applications of cytokine antibody arrays

Currently, cytokine antibody arrays are mainly used for cytokine profiling. However, cytokine antibody arrays can be used for other purposes as shown in **Figure 2**. For instance, cytokine antibody arrays can be used for the identification of cytokine-binding proteins, such as cytokine receptors [78]. In this approach, cytokine antibody chips are incubated with samples containing cytokine and cytokine-associated protein. Antibodies on the chip specifically capture the cytokine and its associated protein. The chip then exposes to the detection antibody specific against cytokine-associated protein. The signals indicate the potential interaction between cytokines and cytokine-associated protein. This approach has been used to study the TNF-α signal transduction pathway and to identify stat1 as a component of the TNF receptor 1 (TNF-R1) and TNF-R1-associated death domain protein signalling complex.

In addition, cytokine antibody arrays may be used to detect autoantibodies. The principle for detection of autoantibodies using cytokine antibody arrays is similar to detecting cytokine-associated protein. As shown in **Figure 2**, cytokine/autoantibody complex are captured onto cytokine antibody chips by cytokine antibody. The chips are then incubated with antibodies against the certain species, such as antibodies against human IgG if human serum or plasma is used..

Finally, cytokine antibody arrays may be used to detect potential modification of cytokines at different situations.

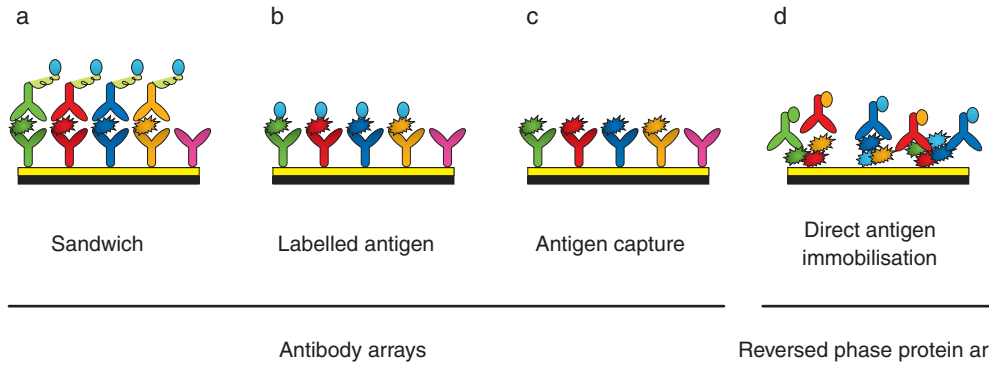

Figure 1. Several immunoassay strategies can be used in cytokine antibody arrays to simultaneously detect multiple cytokines. A) In a sandwich immunoassay, cytokines are captured by antibodies arrayed onto the surface of a solid support and detected by a second labelled antibody. **B)** In labelled antigen assays, cytokines are labelled with certain detection molecules and the cytokines captured by cytokine antibody chips can be detected directly. **C)** In an antigen capture approach, the binding of cytokines to their cognate antibodies will lead to a chemical or physical change, which can be detected using different means. **D)** In reversed phased protein assay, the samples containing a mixture of cytokines are immobilised as an array onto the surface of a solid support. Multiple cytokines can be visualised using a cocktail of antibodies labelled with distinguishable fluorophores.

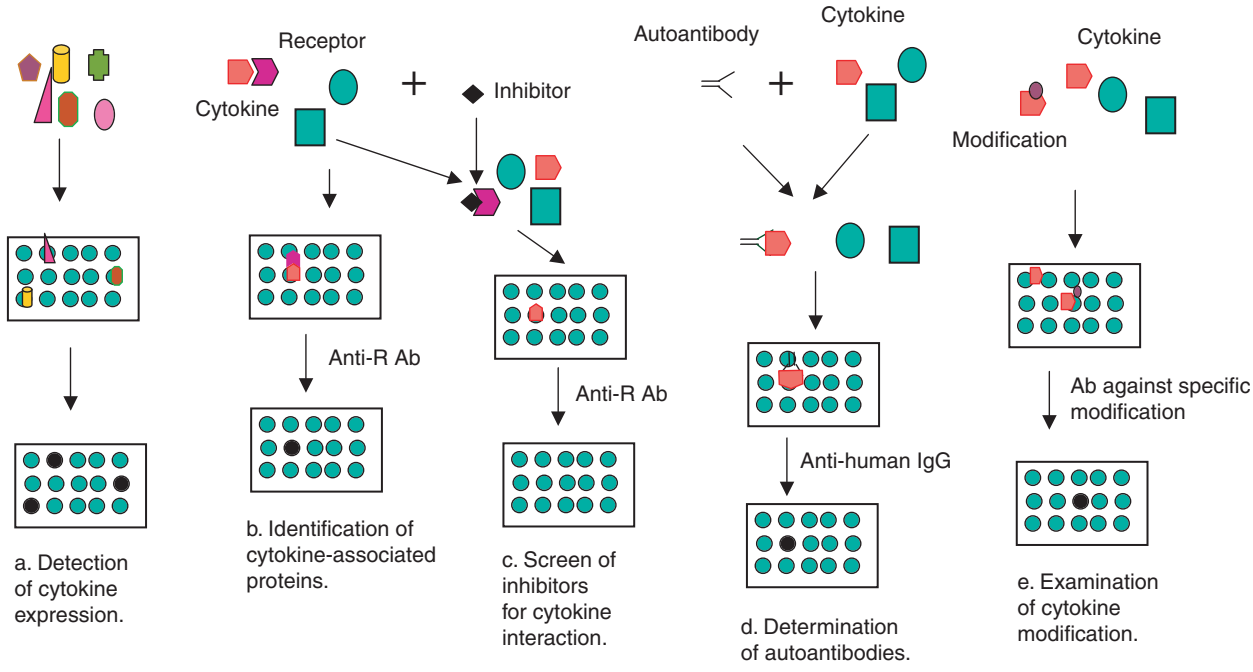

Figure 2. Potential applications of cytokine antibody arrays. A) Detection of cytokine expression. Antibody array chips are incubated with samples. Bound cytokines are detected using detection antibodies. **B)** Identification of cytokine-associated proteins. Samples are exposed to cytokine antibody chips. Cytokines/cytokine-associated proteins are captured by antibody against cytokine(s). The cytokine-associated protein is visualised by detection antibody against the cytokine-associated protein. **C)** Screen of inhibitors for cytokine interaction. The experiments are carried out as in B in the presence of inhibitors. **D)** Determination of auto-antibodies. Cytokine antibody chips are exposed to samples such as human serum. Cytokine/auto-antibody complexes are bound to specific antibodies arrayed on the chips. To detect the existence of auto-antibodies, the chips are incubated with antibody against human IgG. **E)** Examination of cytokine modifications. Cytokines are captured in cytokine antibody chips. The potential modifications of bound cytokines are detected using antibodies against specific modifications.

As shown in **Figure 2**, samples are incubated with cytokine antibody arrays and the modified proteins are detected by antibodies against specific modifications. In theory, this is the easiest way to detect modifications of cytokines. In reality, because modifications do not play major roles in the modulation of cytokine activities for most cytokines as our current knowledge indicates, and only few good antibodies against modifications are available, this approach has not been reported to detect modifications of cytokine in the literature.

6.3 Behind the successful stories

With the commercial availability of cytokine antibody array kits, the applications of cytokine antibody arrays in drug discovery will become increasingly common. More success stories will be heard. However, some failures will also be told. To successfully apply cytokine antibody arrays in the drug discovery process, researchers must bear several important things in mind.

6.3.1 Understanding the advantages and limitations of cytokine antibody arrays

The major advantage of cytokine antibody arrays is that they measure multiple cytokines (currently up to ~ 200) in minute amounts of sample. The major limitation is the difficulty in designing arrays that detect high and low abundances of cytokines simultaneously. The detection sensitivity of cytokine antibody arrays is not exactly the same as ELISA. In some cases, cytokine antibody arrays feature higher detection sensitivity than ELISA. In other cases, there is a lower detection sensitivity in cytokine antibody arrays compared with ELISA.

6.3.2 Setting up a strategy for a project

The best strategy in the opinion of the authors is to screen for cytokine expression using the highest density arrays. From the initial screen, usually one can identify several key factors. Now the focus should be on a more thorough study of these key factors.

6.3.3 Using high-quantity samples

Because cytokines can become degraded during inappropriate storage, care must be taken to minimise the degradation of cytokines. Particularly for clinical samples, it is better to consult a statistician and have pair control samples as well as enough numbers of subjects to generate the data with statistical difference.

6.3.4 Validating some of key data using another independent method

According to the majority of published papers, the data generated from cytokine antibody arrays are in agreement with more conventional approaches. However, when performing high-density arrays, it is highly recommended to independently confirm some of the key data using other approaches, for example, ELISA, Elispot, western blot, or immunostaining.

6.3.5 Extending the data beyond the arrays

As the technology develops further, there will be more high-density and high quality cytokine antibody array products available and exponentially apply those technologies. However, the aim of the authors is to answer some biological questions. Array data definitely will narrow the search for particular candidates and generate new hypotheses but to extend the array technology to biological significance, one usually needs to design a series of experiments and use more traditional approaches to further test the hypotheses. Array data may be a turning point or a starting point for a project (see **Figure 3**). If the technology can be used to go beyond the array data and find biological meaning, the array data will be more fruitful.

6.3.6 Getting meaning from statistics and bioinformatics analysis

Particularly for biomarker discovery, statistics and bioinformatics are the key for success. This type of project usually involves analysis of hundreds and thousands of biological samples. To extract significance from mass data generated from the arrays, great attention must be paid to the low expression cytokines when their expression levels are close to the background. Therefore, certain data must be filtered out for further analysis. Cutoff levels can be defined using various criteria and depend on the overall background levels [79].

Normalisation of different cytokine antibody chips, particularly when experiments are not carried out at the same time, is another important step to generate high quantity data sets for further analysis. Depending on the experimental design, normalisation can be carried out in different ways.

Many cDNA microarray data analysis tools can be used for data generated from cytokine antibody arrays such as cluster analysis [80] and supervised learning [80,81]. This is a growing field that may be taken into consideration when analysing data.

6.3.7 An integrated approach

Cytokines do not act alone; rather they function as an integrated part of biology. They usually bind to their receptors to trigger signal transduction. The changes in cytokines expression can occur at multiple levels, such as the DNA level, the transcription level, the translation level and post-translation level. Therefore, measurement of cytokine protein levels is the only one important investigation of cytokines. To more completely understand the mechanism of cytokines in normal physiology and disease development, an integrated approach involving the determination of cytokine gene, mRNA, protein, structure, associated protein and autoantibody should be used (**Figure 4**).

6.4 Conclusions

Cytokine antibody arrays have gained popularity over the past several years with hundreds of reports in scientific journals and conferences. Numbers of platforms have been

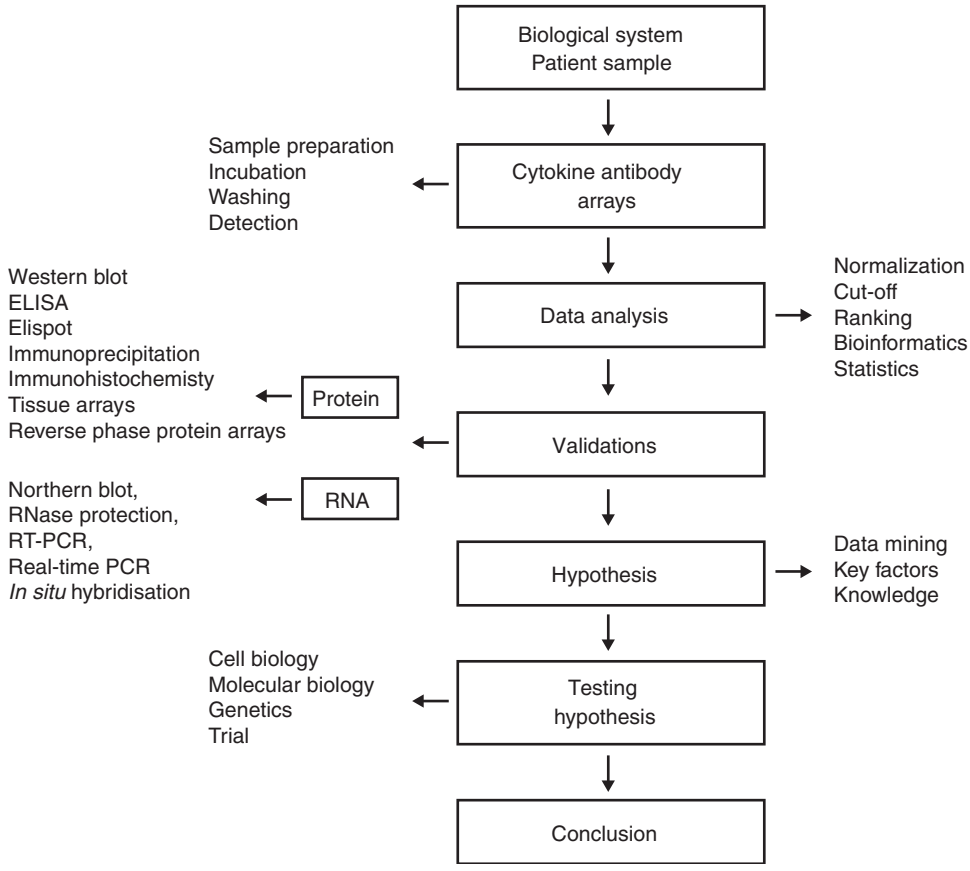

Figure 3. Flow chart of projects using cytokine antibody array technology.
ELISA:Enzyme-linked immunosorbent assay ; PCR: Polymerase chain reaction; RT: Reverse transcriptase.

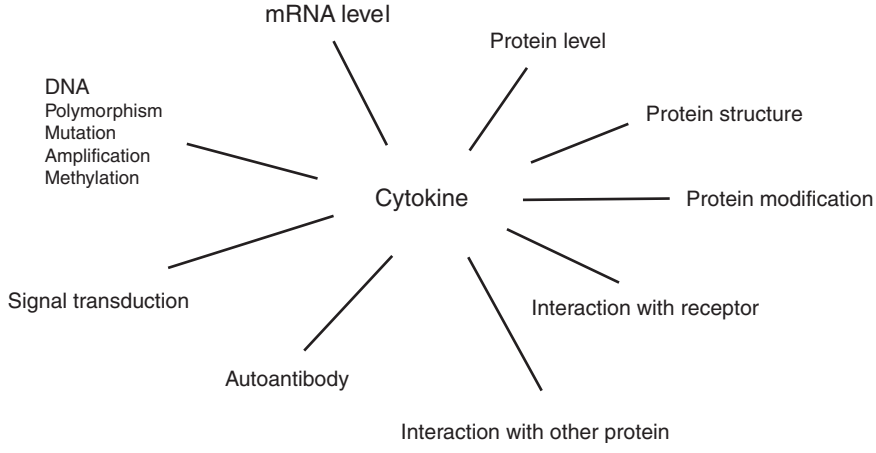

Figure 4. An integrated approach for analysis of cytokines in drug discovery.

established and the kits for detection of multiple cytokines using cytokine antibody arrays have become available from dozens of biotechnology companies. The most common approach for detection of cytokines is the sandwich-based approach. The cytokine antibody arrays have been used in many different labs on a variety of projects with different biological samples, including serum, plasma, conditioned medium, cell lysate, tissue lysate, laser-captured microdissected tissues and CSF. As time passes by, there will be a more in-depth application of cytokine array antibody arrays in drug discovery and more success stories will emerge.

The technology that has been driven by DNA arrays and immunoassays is proving to be quite fruitful. With continuing improvement of the technology and development of capture reagents, the authors anticipate a tremendous growth of cytokine antibody array technology in the years to come. A complete detection of entire cytokine network in single array experiments still need to wait for the emergence of advanced technology. Rather, we anticipate the proliferation of target antibody arrays to detect several dozens of cytokines and cytokine-related protein in the near future. Cytokine antibody arrays will gradually become a routine experimental procedure in the laboratory. Cytokine antibody arrays are a pioneering technology that will have a profound impact on the development of other pathway-focused antibody arrays to detect many other families of proteins. This promising technology will grow beyond the detection of cytokines.

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